

1 Females of the cellar spider discriminate against previous mates

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25 **Abstract**

26

27 Mate choice for novel partners should evolve when re-mating with males of varying genetic
28 quality provides females with fitness-enhancing benefits. We investigated sequential mate choice
29 for same or novel mating partners in females of the cellar spider *Pholcus phalangioides*
30 (Pholcidae) to understand what drives female re-mating in this system. Females are moderately
31 polyandrous and show high reluctance to re-mating. Yet, double-mated females benefit from a
32 higher oviposition probability compared to single-mated females. We exposed mated females to
33 either their former (same male) or to a novel mating partner, and assessed mating success
34 together with courtship and copulatory behaviours in both sexes. We found clear evidence for
35 mate discrimination: females experienced three-fold higher re-mating probabilities with novel
36 males, being more often aggressive towards former males and accepting novel males faster in the
37 second than in the first mating trial. Preference for novel males suggests that re-mating is driven
38 by benefits derived from multiple partners. The low re-mating rates and the strong last male
39 sperm precedence in this system suggest that mating with novel partners that represent
40 alternative genotypes may be a means for selecting against a former mate of lower quality.

41

42 **Introduction**

43

44 Litters, clutches and broods are commonly sired by more than one male (Birkhead & Møller
45 1998; Taylor et al. 2014) suggesting that, despite the substantial costs incurred from re-mating
46 (Knell & Webberley 2004; Arnqvist & Rowe 2005), polyandry (female multiple mating) is a
47 pervasive mating strategy. Addressing female interests in the study of mating system evolution
48 provides insight into its adaptive explanations (Arnqvist & Nilsson 2000; Jennions & Petrie
49 2000; Hosken & Stockley 2003). By mating with multiple males females may enhance the
50 chances of siring offspring of higher genetic quality or with optimal genetic compatibility
51 (genetic benefits models) (Newcomer et al. 1999; Tregenza & Wedell 2002). Females may also
52 derive fecundity benefits by receiving male-derived resources as food, parental care or sperm
53 (material benefits models) (Arnqvist & Nilsson 2000). Polyandry may nevertheless also be the
54 non-adaptive outcome of enforced matings with males that are able to overcome female
55 reluctance to mate (sexual conflict models) (Holland & Rice 1998; Arnqvist & Rowe 2005).

56

57 Investigating mate choice for novel or previous mates may provide a useful tool to
58 understand the underlying adaptive explanations for female re-mating. If females receive
59 material fitness-enhancing benefits, for instance sperm supplies, selection should promote
60 indiscriminate repeated matings regardless of whether they occur with the same or different
61 mating partners (Ridley 1988; South & Lewis 2011). On the contrary, females can only assure
62 genetic benefits by varying the genetic quality of their mates to allow post-mating selection for
63 best or most compatible genotypes through sperm competition or female fertilization biases
64 (Jennions & Petrie 2000; Simmons 2001; Bretman et al. 2009). In the latter case females are
65 expected to avoid re-matings with previous mates. Indeed, in hamsters (Lisk & Baron 1982),
66 guppies (Hughes et al. 1999; Eakley & Houde 2004), hide beetles (Archer & Elgar 1999),
67 crickets (Bateman 1998; Ivy et al. 2005), dung flies (Hosken et al. 2003) and pseudoscorpions

68 (Zeh et al. 1998), polyandrous females are known to mate preferentially with novel males when
69 given the opportunity.

70
71 Females can acquire better or more compatible genes for their offspring without
72 necessarily enhancing post-mating selection mechanisms. In systems where last males to mate
73 experience highest fertilization success (hereafter called last male sperm precedence), as some
74 insects and arachnids (Birkhead & Hunter 1990; Elgar 1998), female mate choice for novel
75 mating partners will inevitably select against previous mates. In such systems, females may gain
76 benefits for their offspring by re-mating exclusively when they encounter a male of superior
77 quality compared to their previous mate, polyandry being used as a strategy to compensate for
78 lower quality inseminations.

79
80 In this study we investigated female sequential mate choice for same or novel mating
81 partners in the cellar spider *Pholcus phalangioides* (Pholcidae), a cosmopolitan web-building
82 spider whose reproductive behaviour has been extensively studied (Uhl 1998; Schäfer & Uhl
83 2002; Schaefer & Uhl 2003; Schäfer & Uhl 2005; Uhl et al. 2005). Females are moderately
84 polyandrous (83.3% of wild-caught broods are sired by more than one male, with an average of
85 2.5 sires, Schultz and Uhl unpublished) even though one mating is sufficient to produce fertile
86 egg sacs throughout the female's lifetime (Uhl 1993). Laboratory studies show that females do
87 not readily re-mate: female acceptance of first matings is close to 100% but drops to 68-82% in
88 second matings (Schäfer & Uhl 2002; Schäfer & Uhl 2005; Schäfer et al. 2008). Males cannot
89 enforce copulations, because females need to position themselves horizontally and expose their
90 genital opening, so that males can secure their mouthparts to a sclerotized hook of the female's
91 genital plate and introduce both of their pedipalps (Uhl et al. 1995). The reproductive interests
92 over re-mating between the sexes appear to be in conflict. Due to last male sperm precedence,

93 second males to mate fertilizing 88-89% of the offspring (Schäfer & Uhl 2002; Schäfer et al.
94 2008), so it is in the males best interest to mate with already mated females.
95 Despite female reluctance to accept second mates, re-mating is known to be beneficial for female
96 fitness, double-mated females experiencing higher oviposition probabilities compared to single-
97 mated females (Uhl et al. 2005). What remains unclear is whether re-mating triggers oviposition
98 through the reception of more sperm and/or stimulants in the ejaculate (direct benefit models),
99 which could also be achieved by mating repeatedly with the same male, or through the effect of
100 multiple ejaculates (genetic benefit models) (Tregenza & Wedell 1998).

101
102 We use female choice for novel or former mating partners to shed light on the adaptive
103 explanation for polyandry in this system, as mate choice for male novelty should evolve
104 exclusively when fitness benefits derive from multiple mates. We exposed mated females
105 sequentially to either the same male as in their first mating (same male treatment, SM) or to a
106 novel male, which the female had never encountered (novel male treatment, NM) and scored
107 differences in female pre-copulatory (aggressive interactions, acceptance of the mate, and
108 latency to copulation) and copulatory behaviours (copulation duration) which were used as
109 proxies for female mate choice. To control for differences in male mating effort towards novel or
110 previous females we also scored male pre-copulatory (latency to courtship, coupling attempts)
111 and copulatory (pedipalp movements) behaviours. If re-mating is beneficial due to the reception
112 of multiple partners we expect females to mate preferentially with novel mates, whereas if re-
113 mating is necessary to obtain sufficient sperm or sufficient oviposition stimulants females should
114 re-mate regardless of male novelty.

115

116 **Methods**

117

118 **Collection, rearing and body measures**

119 Immature spiders during their penultimate (4th and 5th) sub adult stages were collected from
120 several buildings in the city of Barcelona (Spain) and brought to the laboratory during spring
121 2008. Individuals were reared individually in (length, width, height) 16 x 9 x 9 cm transparent
122 containers and were fed five *Drosophila melanogaster* twice a week and two crickets *Acheta*
123 *domestica* once a week. Upon reaching adulthood, spiders were sexed and the tibia-patella length
124 of the first right leg was measured and used as a proxy for body size (Schäfer et al. 2008). Each
125 individual was anaesthetized with CO₂, placed onto a squared-millimeter paper with the legs
126 parallel to the surface and a photograph was taken with a Canon EOS 350D camera. Images were
127 then processed with SigmaScan Pro 5.0.0 software to obtain the body measurements. All
128 individuals (and their offspring) were released after the end of the study, in September 2008.

129

130 **Experimental design**

131 Virgin females were randomly assigned to either of the two treatments, “same male” (SM,
132 N=38) or “novel male” (NM, N=37). All females were first mated with a virgin male and were
133 subsequently presented with a second male in order to re-mate. SM females were given the same
134 male as in their first mating, whereas NM females a novel male which had previously mated to a
135 virgin female of the same treatment. Hence, both NM and SM males, in the second mating, had
136 the same mating status (mated once with a virgin female) (Tregenza & Wedell 1998).

137

138 Since body size differences within a mating pair are known to affect copulatory behaviours
139 in this species (Schäfer et al. 2008), males were assigned to females with matching patella-tibia
140 length (± 0.3 mm, see (Schäfer & Uhl 2002)). There were no significant differences in tibia-
141 patella lengths (mm) between treatments in either females (mean \pm SE) (SM = 11.70 ± 0.27 ; NM
142 = 12.15 ± 0.17 ; $F_{1,73} = 1.99$, $p = 0.16$) or males (SM = 11.68 ± 0.27 ; NM = 12.14 ± 0.17 ; $F_{1,73} =$
143 1.95 , $p = 0.17$).

144

145 All spiders were mated within 45 days after their final moult to adulthood to prevent age
146 affecting copulatory behaviour, as it is known that number of PPM decreases with male age
147 (Schäfer & Uhl 2002). There were no significant differences in age (number of days) between
148 treatments in either females (SM = 33.82 ± 1.49 ; NM = 32.60 ± 1.41 ; $F_{1,73} = 0.36$, $p = 0.55$) or
149 males (SM = 29.45 ± 1.78 ; NM = 31.70 ± 1.53 ; $F_{1,73} = 0.92$, $p = 0.34$). Individuals of a mating
150 pair were of the same age (females = 33.21 ± 1.02 ; males = 30.56 ± 1.18 ; paired t-test: $t_{74} = 1.66$,
151 $p = 0.1$). Females were transferred to a new container (14.5 x 9 x 6.5 cm) 12 hours before the
152 scheduled mating to give them time to spin a web and acclimatize to the new environment.
153 Mating boxes were only used a single time to prevent intermixing of chemical cues between
154 mating trials. Thus, 150 mating boxes were used in total. Females of each treatment were given a
155 second male between 3 and 5 hours after the first mating because female re-mating probability
156 drops steeply after 6 hours (Schäfer & Uhl 2005). This also allows males to perform recharge
157 their pedipalps with sperm after the first copulation. A sub-sample of males (N = 20) was
158 constantly monitored through direct observations and/or video recording to document male
159 recharge of pedipalps. Sperm induction in *P. phalangioides* has been described as males hanging
160 upside down in their web and taking the seminal drop with the third pair of legs; the drop is
161 placed between the chelicerae and then the male dips the pedipalps alternatively into the drop to
162 charge them (Gerhardt (1927) in Huber (1998)).

163

164 **Behavioural observations**

165 Courtship is almost always initiated by the male. Males can begin courtship with different
166 behaviours, such as performing abdominal vibrations, tapping and jerking the female's web, and
167 tapping the female's legs using his first pair of legs (Bartos 1998). Females do not move during
168 courtship, though they can produce web vibrations. We considered courtship to start upon the
169 first contact of a male with the female because the other behaviours are difficult to detect and can
170 be missing from the courtship sequence, whereas tapping of the female's legs always happens

171 (Bartos 1998). In both first and second mating trials we scored the following pre-copulatory
172 variables: latency to courtship, defined as the time from the start of the experiment to the first
173 physical contact between a male and a female; occurrence of aggressive interactions (yes/no),
174 defined as females rejecting males by pushing the male away with front leg-movements and/or
175 chasing the male; latency to copulation, defined as the time from courtship to copulation; and
176 number of coupling attempts, since males typically perform several attempts before introducing
177 their pedipalps into the genital cavity and start copulation. While latency to courtship and
178 coupling attempts are behaviours under male control, we use latency to copulation and
179 aggressive interactions as proxies for female willingness to mate. A mating trial began when a
180 male was introduced into the female box. The pair was then given 90 minutes to start copulation
181 (Schäfer & Uhl 2002). If the pair did not mate within this timeframe, the male was removed from
182 the box and the mating trial scored as failed. The copulatory variables scored were number of
183 pedipalp movements (PPM) and copulation duration. PPMs are rhythmic movements that males
184 make during copulation with both inserted pedipalps and that correlate positively with the
185 amount of sperm transferred (Uhl unpublished) and paternity success (Schäfer & Uhl 2002). All
186 successful copulations were observed until male pedipalps became detached from the female
187 genitalia.

188
189 For those males monitored during sperm induction we recorded: latency to sperm
190 induction, defined as the time elapsed from the first copulation until start of sperm uptake;
191 induction duration, defined as the total time spent recharging its pedipalps; and number of sperm
192 uptake movements. We considered induction to start when the third pair of legs was bent to
193 contact with the genital pore and we considered it to end when the male un-bended the legs. All
194 behavioural observations were done by LM.

195

196 **Statistical analyses**

197 We used generalized linear models (GLM) with Gamma (GLM-g) distribution for continuous
198 variables (latency to courtship, latency to copulation, copulation duration), Poisson (GLM-p)
199 distribution (log-link function, corrected for overdispersion) for count variables (number of
200 coupling attempts, number of PPM) and Binomial (GLM-b) distribution (logit-link function,
201 corrected for overdispersion) for dichotomous variables (occurrence of aggressive interactions),
202 and ran the models on the effect of treatment (SM or NM) for the first mating to ensure that
203 behaviours scored did not differ. Treatment effects (SM, NM) on female re-mating decisions
204 (accept/reject second copulation) and occurrence of aggressive interactions (yes/no) were
205 analysed with GLM using the Binomial (GLM-b) distribution (logit-link function, corrected for
206 overdispersion). In all these models that tested treatment effects we included “body size
207 difference” (absolute value of the body size difference between the members of the mating pair)
208 and “age” (age of the oldest individual of the mating pair) and their interactions with treatment to
209 control for the potentially confounding effects of these variables (see “Experimental design”
210 above). Finally we employed generalized linear mixed models (GLMM) to account for female
211 individual autocorrelation, with the above-mentioned error distributions, and ran the model on
212 the effect of mating trial number (first and second mating) on mating behaviours within each
213 treatment (SM and NM), including “body size difference” and “age” as covariates. We always
214 started with full models and used manual stepwise backward selection to discard nonsignificant
215 effects, which led to final models only including the effects of treatment or of mating trial
216 number, or to no effects at all. We conducted all analyses using R version 3.1.0 (R Development
217 Core Team 2014), with the package MASS for mixed models (Venables & Ripley 2002). Results
218 are shown as the mean \pm SE.

219

220 **Results**

221 **First mating trials**

222 All virgin females mated with the first male. None of the behavioural variables differed between
223 SM and NM treatments (GLM-b, occurrence of aggressive interactions = 0.07 ± 0.03 ; GLM-g,
224 latency to courtship = 2.94 ± 0.74 minutes, latency to copulation = 19.48 ± 2.42 , copulation
225 duration = 80.22 ± 3.81 minutes; GLM-p, number of coupling attempts = 15.60 ± 2.96 , number
226 of PPM = 185.00 ± 6.98 , all $p > 0.21$).

227

228 **Second mating trials**

229 Re-mating probability in NM females was more than three times as high as in the SM treatment:
230 only 3 out of 38 females of the SM treatment re-mated, compared to 10 out of 27 females of the
231 NM treatment (GLM-b, $\chi^2_{1,73} = 64.17$, $p = 0.025$; **Fig. 1**), and the probability of a female being
232 aggressive towards the male was more than four times as high in the SM as in the NM treatment
233 (SM = 0.75 ± 0.074 , NM = 0.16 ± 0.061 ; $\chi^2_{1,71} = 73.29$, $p < 0.0001$).

234

235 There was no difference between treatments in male latency to courtship (GLM-g, $F_{1,70} =$
236 2.57 , $P = 0.11$; **Fig. 2a**). However, comparisons of first and second mating trials showed that
237 males initiated courtship sooner when they were presented the same female a second time, as
238 latency to courtship was lower in second than in first matings in the SM treatment ($F_{1,34} = 8.41$,
239 $p = 0.0065$) but not in NM males ($F_{1,36} = 0.14$, $p = 0.71$; **Fig. 2b**).

240

241 In the NM treatment, all copulatory behaviours of males and females differed between first
242 and second mating trials: mated females accepted males faster but copulated for a much shorter
243 time, whereas mated males performed a higher number of coupling attempts but did less PPMs
244 than in the first mating (**Table 1**). All second copulations in both SM and NM treatments were
245 short [< 47 PPM, *sensu* (Schäfer et al. 2008)], except for one NM copulation with 189 PPM that
246 lasted for 79.78 minutes, which was excluded from data analysis when comparing first and
247 second matings. Due to the low number of SM females that accepted to re-mate ($N = 3$), number

248 of coupling attempts and copulatory variables between first and second matings in this treatment
249 group are not compared.

250

251 **Sperm induction**

252 Males initiated post-mating sperm induction one hour and a half after the first copulation
253 (induction latency: 88.72 ± 3.94 minutes, $N = 20$). Sperm induction lasted on average $5.13 \pm$
254 0.30 minutes ($N = 17$) and included 7.17 ± 0.83 sperm uptake movements ($N = 12$).

255

256 **Discussion**

257 Our study revealed that *Pholcus phalangioides* females discriminate against previous mates
258 when given the opportunity to re-mate. Females exposed to novel males exhibit three-fold higher
259 re-mating probabilities than those encountering former mating partners. In accordance to such
260 finding, females were more aggressive towards former mates, rejecting and chasing former
261 males in higher proportions compared to males that had not been encountered previously. Novel
262 males presented in second mating trials were also accepted sooner than virgin novel males
263 presented in first mating trials. Altogether our data show that females prefer re-mating with
264 novel males.

265

266 Although we could not test for reproductive success differences between treatments due to
267 the very low numbers of females accepting a second mate, female mate choice patterns may
268 indirectly shed light on the adaptive explanations for re-mating in this system. Preference for
269 novel males implies that fitness benefits of re-mating arise from the ability to select for multiple
270 different partners. Due to *P. phalangioides* low re-mating rates (27% in the NM treatment) and
271 last male sperm precedence —second males to mate are known to mechanically remove sperm
272 (i.e. eject or translocate) from previous males via pedipalp movements (Schäfer & Uhl 2002),
273 their intromittent organs accessing directly female sperm storage sites (Uhl 1994)— it is unlikely

274 that polyandry is a means to promote post-mating processes selecting for best or most
275 compatible genes (Jennions & Petrie 2000; Simmons 2001). Mating with a novel male may
276 prove beneficial if the male's quality exceeds the quality of a previous mate, mate choice
277 predicting fertilization outcome. Hence, previously reported higher oviposition probabilities of
278 double- compared to single-mated *P. phalangioides* females (Uhl et al. 2005) may have been
279 triggered by mating with a novel mating partner and not by the number of matings *per se*. The
280 novel mate may overcome low quality inseminations from males unsuccessful in triggering
281 oviposition due to infertility, failure of sperm transfer or due to lack of oviposition stimulants in
282 their ejaculates (Jennions & Petrie 2000). Insect males are known to transfer substances in their
283 seminal fluids which are able to stimulate ovulation by enhancing female production of egg-
284 laying hormones (Lange 1984; Stanley 2006), or by directly interfering with females neuronal
285 pathways (Rubinstein & Wolfner 2013). In addition, since seminal fluids are complex mixtures
286 of molecules exhibiting large between-male variation in their composition (Poiani 2006) it may
287 be the synergistic effect of multiple stimulants inside different ejaculates to ultimately trigger
288 oviposition (Eady et al. 2000). Although seminal fluid composition has not yet been
289 characterized in spiders, similar processes are likely to occur, and positive effects of multiple
290 ejaculates on oviposition probabilities have been documented in this group (Tuni et al. 2013).

291
292 Unexpectedly, the re-mating rate of females with novel males in our study (27%) was
293 much lower than that reported in previous studies (> 68%) despite similar experimental
294 protocols (Schäfer & Uhl 2002, 2005; Uhl et al. 2005; Schäfer et al. 2008). The only difference
295 is that previous studies used virgin males in second mating trials, whereas we used once-mated
296 males. This suggests that females may prefer mating with virgin than with non-virgin males, a
297 strategy possibly adopted to avoid males with declining reproductive output following
298 consecutive matings (i.e. sperm depletion) (South and Lewis 2011), or reduce the risk of sexually
299 transmitted disease infection (Knell and Webberley 2004).

300
301 Sexual interest in males is known to decline after repeated matings with the same female,
302 while it restores when experiencing novel mating partners (Wilson et al. 1963; Dewsbury 1981).
303 Such phenomenon, known as ‘Coolidge effect’, is driven by prudent allocation of costly male
304 resources (sperm and seminal fluids) based on female quality (Wedell et al. 2002). Low sample
305 sizes prevented us from comparing number of pedipalp movements between treatments which, as
306 indicators for sperm allocation (Schäfer & Uhl 2002), would allow full detection of differences
307 in male investment. Nevertheless, by comparing the latency of male initiation of courtship
308 between treatments we can exclude that the higher re-mating probability with novel partners is
309 due to greater male attraction to novel females (Gershman & Sakaluk 2009; Tuni & Bilde 2010;
310 Werminghausen et al. 2013) (but see (Steiger et al. 2008)). On the contrary, males started
311 courting former mates sooner compared to novel ones. Thus, males may be able to recognize
312 previously mated females, for example via pheromones (Gaskett 2007), and adjust their
313 behaviour accordingly. This could be interpreted as a strategy for males to reduce the costs of
314 mating when exposed to a new individual (i.e. disease or parasite transmission) (Daly 1978;
315 Kokko et al. 2002)

316 We conclude that females of the cellar spider discriminate against previous mates. The
317 fitness benefits to choosy females should select for mate recognition, though the mechanisms
318 through which females discriminate males remain unexplored. Complex vibratory and/or
319 chemical signals may operate in concert while males approach females on their webs allowing
320 females to assess male identity (Huber 2005).

321

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327

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445 **Figure Legends**

446

447 **Fig. 1.**

448 Female re-mating probability when exposed to same (SM) or novel (NM) male. Results are
449 shown as the mean \pm SE.

450

451 **Fig. 2.**

452 Male latency to courtship during (a) same (SM) and novel (NM) male treatment and (b) during
453 first and second mating trials in the SM treatment. Results are shown as the mean \pm SE.

454 **Table 1.**

455 Comparison of pre-copulatory and copulatory behaviours between first and second mating trials
456 with novel males (NM treatment).

457

| Variable | First mating | | Second mating | | N | GLMM |
|-----------------------------|--------------|-------|---------------|-------|----|-------------------------------|
| | mean | SE | mean | SE | | |
| Courtship latency (min) | 2.57 | 1.19 | 2.12 | 0.75 | 37 | $F_{1,36} = 0.14, p = 0.71$ |
| Copulation latency (min) | 17.36 | 5.60 | 6.61 | 1.53 | 9 | $F_{1,8} = 6.49, p = 0.034$ |
| Number of coupling attempts | 10.33 | 1.89 | 28.67 | 18.77 | 9 | $F_{1,8} = 6.78, p = 0.032$ |
| Copulation duration (min) | 93.12 | 10.42 | 1.30 | 0.63 | 9 | $F_{1,8} = 7.97, p = 0.022$ |
| Number of PPM | 190.22 | 12.92 | 10.33 | 4.62 | 9 | $F_{1,8} = 56.47, p = 0.0001$ |

458

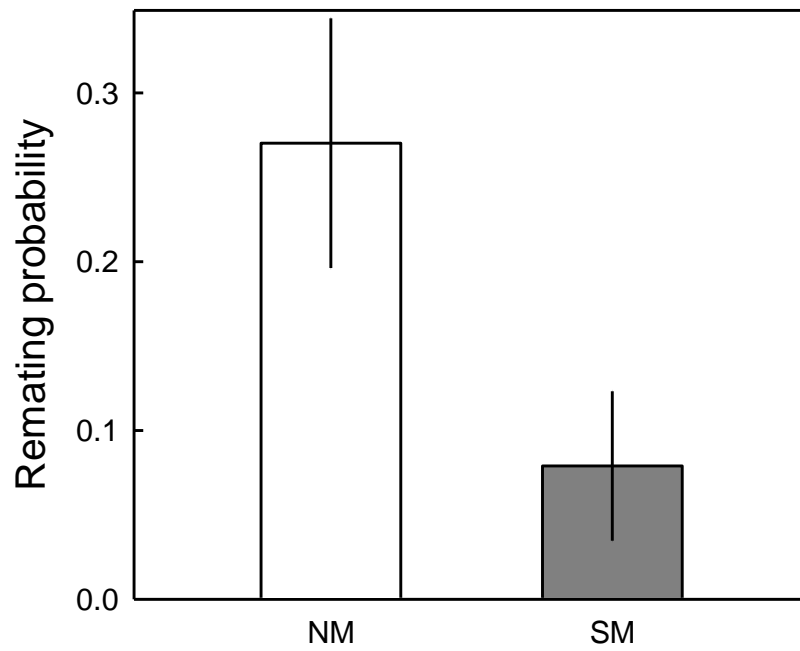
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460

461 **Fig. 1.**

462 Female re-mating probability when exposed to same (SM) or novel (NM) male. Results are

463 shown as the mean \pm SE.

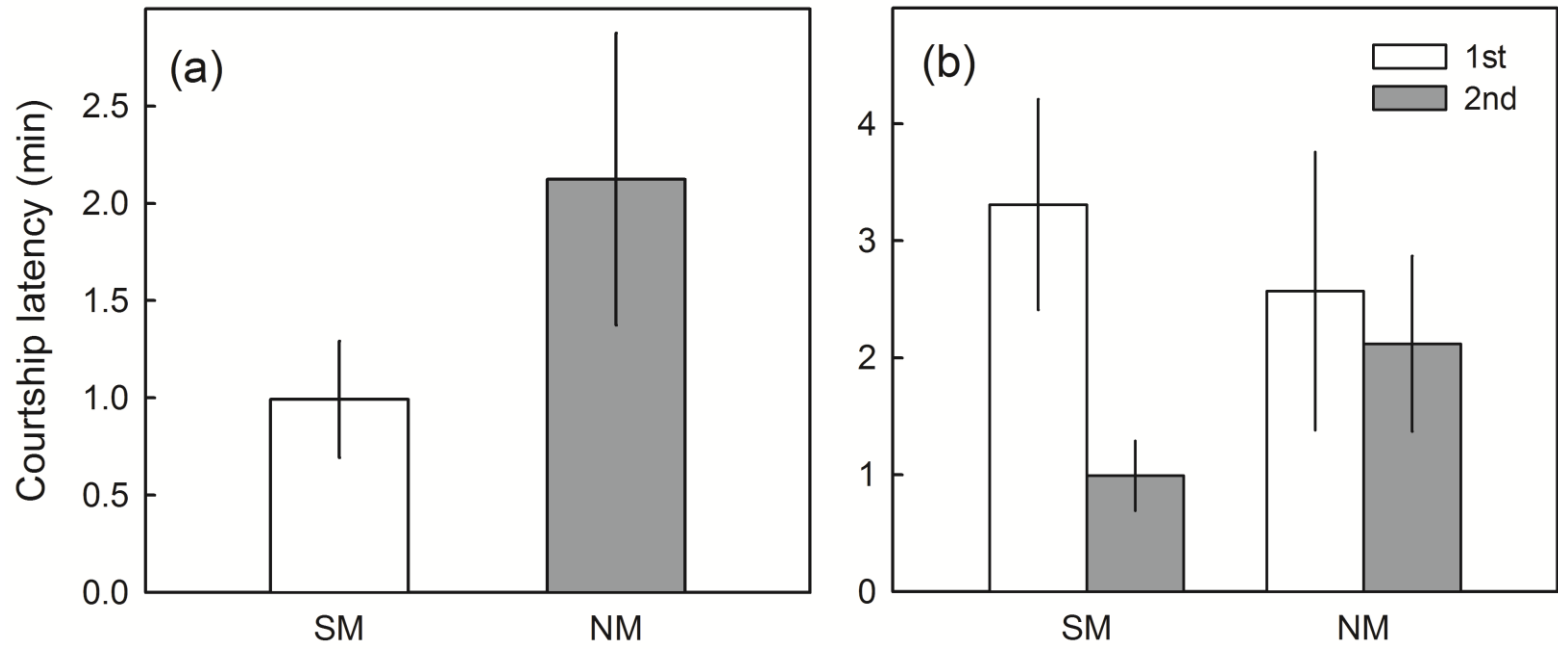


464

465

466 **Fig. 2.**

467 Male latency to courtship during (a) same (SM) and novel (NM) male treatment and (b) during
468 first and second mating trials within each treatment. Results are shown as the mean \pm SE.



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